

PCT

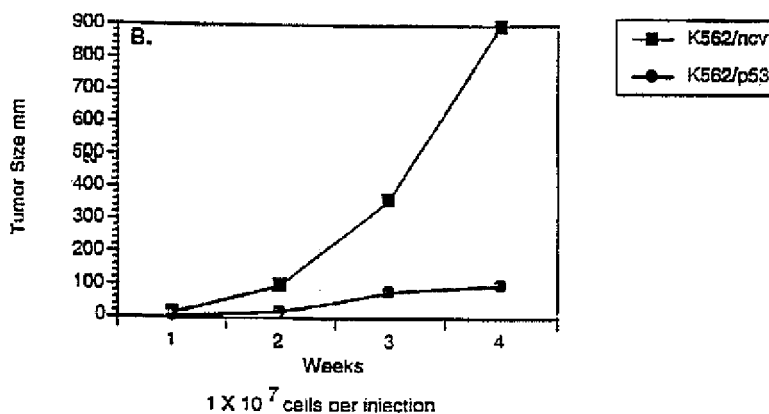
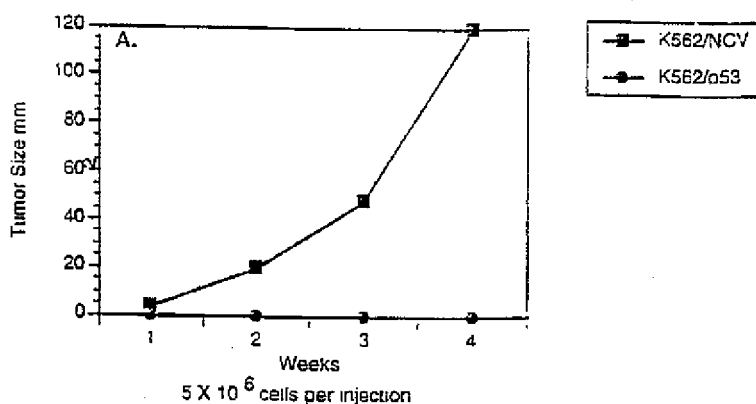
WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 15/12, A61K 48/00</b> <b>C07K 13/00, C12N 15/86</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/06910</b> <b>(43) International Publication Date:</b> 31 March 1994 (31.03.94)
<b>(21) International Application Number:</b> PCT/US93/08844 <b>(22) International Filing Date:</b> 17 September 1993 (17.09.93) <b>(30) Priority data:</b> 07/948,289 18 September 1992 (18.09.92) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 07/948,289 (CIP) Filed on 18 September 1992 (18.09.92) <b>(71) Applicant (for all designated States except US):</b> CANJI, INC. [US/US]; 3030 Science Park Road, Suite 302, San Diego, CA 92121-0177 (US).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> SHEPARD, H., Michael [US/US]; 2745 Argonauta Street, La Costa, CA 92009 (US). KAN, Nancy [US/US]; 4417 Camden Circle, Dublin, OH 43017 (US). <b>(74) Agents:</b> KONSKI, Antoinette, F. et al.; Campbell & Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US). <b>(81) Designated States:</b> AU, CA, JP, KR, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

**(54) Title:** GENE THERAPY BY RETROVIRAL VECTOR WITH TUMOR SUPPRESSIVE GENE**(57) Abstract**

A method for transducing a pathologic hyperproliferative mammalian cell is provided by this invention. This method requires contacting the cell with a suitable retroviral vector containing a nucleic acid encoding a gene product having a tumor suppressive function. Also provided by this invention is a method for treating a pathology in a subject caused by the absence of, or the presence of a pathologically mutated tumor suppressor gene.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

## GENE THERAPY BY RETROVIRAL VECTOR WITH TUMOR SUPPRESSIVE GENE.

FIELD OF THE INVENTION

The present invention generally relates to a  
5 method for selectively transducing pathologic  
hyperproliferative mammalian cells in a heterogeneous cell  
preparation comprising retroviral-mediated transduction of  
the pathologic cell with a nucleic acid encoding a gene  
product having tumor suppressive function.

10 Throughout this application various publications  
are referenced within parentheses. The disclosures of  
these publications in their entireties are hereby  
incorporated by reference in this application in order to  
more fully describe the state of the art to which this  
15 invention pertains.

BACKGROUND OF THE INVENTION

The human p53 gene encodes a 53 kilodalton  
nuclear phosphoprotein (Lane, D.P., et al., Genes and Dev.,  
4:1-8 (1990); Lee, Y-HP, Breast Cancer Res. and Trmt., 19:3-  
20 13 (1991); Rotter, V., et al., Adv. Can. Res., 57:257-72  
(1991)). The p53 protein was first identified as a  
cellular protein in SV40-transformed cells that was tightly  
bound to the SV40 T antigen (Lane, D.P., et al. Nature,  
278:261-3 (1979)). The wild type p53 gene has the  
25 characteristics of a tumor suppressor gene. It is similar  
to the prototype of tumor suppressor genes, the  
retinoblastoma gene (RB), in that loss of heterozygosity of  
the p53 or RB genes characterizes the phenotype of many  
types of tumor cells (Hollstein, M. et al., Science,  
30 253:49-51 (1991); Levine, A.J., et al., Biochimica et  
Biophysica Acta, 1032:119-36 (1990); Levine, A.J., et al.,  
Nature 351:453-6 (1991); Weinberg, R.A. Science, 254:1138-  
46 (1991)). In human malignancies associated with p53

alterations, this loss of heterozygosity usually results from the loss of one allele (allelic deletion), while the other allele suffers one or more somatic mutations. Unlike RB, however, certain mutations in the p53 gene are capable of immortalizing rodent cells and enhancing the tumorigenicity of established cell lines (Jenkins, J.R., et al., Nature, 312:651-4 (1984)). Mutant but not wild type p53 can cooperate with the activated ras oncogene in transforming primary rat embryo fibroblasts (Eliyahu, D., et al., Nature, 312(13):646-9 (1984); Parada, L.F., et al., Nature, 312:649-51 (1984)). Other events related to tumor progression also appear to be associated with the expression of mutant p53. Among these is differential modulation of the multiple drug resistance gene (MDR1) by wild type as compared to altered p53. In this case, mutant p53 specifically stimulates the MDR1 promoter, while wild type p53 exerts repression (Chin, K-V., et al., Science, 255:459-62 (1992)). Another possible way in which mutant p53 could promote tumorigenesis is by reducing tumor cell responsiveness to transforming growth factor- $\beta$ , a negative regulator of cell proliferation (Gerwin, B.I., et al., PNAS USA, 89:2759-63 (1992)).

In addition to the *in vitro* data described above two animal models have been described that implicate p53 in tumor formation. Transgenic mice expressing a mutant p53 gene display a high incidence of lung, bone and lymphoid tumors (Lavigne, A., et al. Mol. Cell. Biol., 9(9):3982-91 (1989)). In addition, p53-null mice (Donehower, L.A., et al., Nature, 356(19):215-21 (1992)) show an increased risk of spontaneous neoplasms, the most frequently observed being malignant lymphoma.

Other data which support the conclusion that mutant p53 plays an important role in tumorigenesis include re-introduction of the wild type p53 gene into human tumor cell lines which lack p53 expression. In this case, wild

type p53 can reverse the malignant phenotype as measured by colony formation in soft agar and tumor formation in nude mice (Chen, P.L., et al., Science, 250:1576-80 (1990); Cheng, J., et al., Can. Res., 52:222-6 (1992); Baker, S.J., et al., Science, 249:912-15 (1990); Isaacs, W.B., et al., Can. Res., 51:4716-20 (1991); Casey, G., et al., Oncogene, 6(10):1791-7 (1991); Shaw, P., et al., PNAS USA, 89:4495-99 (1992); Takahashi, T., et al., Can. Res., 52:2340-3 (1992)). Tumor cell types which have shown conversion of a non-malignant phenotype as a result of the introduction of wild type p53 expression include prostate (Isaacs, W.B., et al., supra), breast (Casey, G. et al. supra), colon (Baker, S.J., et al., supra; Shaw, P. et al., supra) lung (Takahashi, T. et al., supra), and lymphoblastic leukemia (Cheng, J. et al., supra). Other data suggest that introduction of wild type p53 into tumor cells which have lost endogenous p53 expression appears to be cytotoxic (Johnson, P. et al., Mol. Cell. Biol., 11(1):1-11 (1991)). In some cases the re-introduction of wild type p53 may result in programmed cell death, or apoptosis (Yonish-Rouach, E. et al., Nature, 352:345-7 (1991)). The work described above indicates strongly that alteration of the wild type p53 gene has a role in multiple aspects of tumorigenesis and that reintroduction of the wild type p53 coding sequence can have a negative regulatory function or cytotoxic effect on malignant cells.

Clinical data suggest that inactivating mutations in the p53 gene are among the most common types of mutations associated with human malignancy (Rotter, V. et al. supra; Nigro, J.M. et al., Nature, 342:705-8 (1989); Gaidano, G. et al., PNAS USA, 88:5413-7 (1991); Cheng, J. et al., Mol. Cell. Biol., 10(10):5502-09 (1990)). A classical example is the Li-Fraumeni syndrome, a familial syndrome of several neoplasms, including breast cancer, sarcomas and others. Specific mutations in the p53 gene are found in affected members of the family and appear to

be associated with the predisposition to develop early cancers (Malkin, D. et al., Science, 250:1233 (1990); Srivastava, S. et al., Nature, 348:747 (1990)). Several laboratories have reported that alterations in the p53 gene  
5 accompany the evolution of human CML (chronic myelogenous leukemia) to blast crisis (acute phase) (Ahuja, H. et al., J.Clin.Invest., 87:2042-7 (1991); Foti, A. et al., Blood, 77(11):2441-4 (1991); Feinstein, E. et al., PNAS USA, 88:6293-7 (1991)). In one CML patient who reverted briefly  
10 from the acute phase to a second chronic phase, the inactivating point mutation in p53 which appeared concomitantly with the acute phase disappeared and the wild type sequence re-emerged (Foti, A. et al., supra). These data indicate that alterations which inactivate the tumor  
15 suppressive activity of p53 may represent pivotal events in the progression from the chronic to the acute phase of human CML. Other clinical data also suggest an important role for p53 mutations in disease progression. These include a number of hematologic neoplasms as well as solid  
20 tumors (Rotter, V. et al. supra; Ahuja, H. et al., J.Clin.Invest., supra; Ahuja, H. et al., PNAS USA, 86:6783-6787 (1989); Mori, N. et al., Br. J. of Haem., 81:235-240 (1992); Porter, P.L. et al., Am.J.Path., 140(1):145-53 (1992)). Recent reports show a dramatic association between  
25 altered p53 and shortened survival in breast cancer (Thor, A.D. et al., J.Natl. Can. Inst., 84(11):845-55 (1992); Isola, J. et al., J.Natl. Can. Inst., 84(14):1109-14 (1992); Callahan, R. J.Natl. Can.Inst., 84:826-7 (1992)).

#### SUMMARY OF THE INVENTION

30 The present invention generally relates to a method for selectively transducing pathologic hyperproliferative mammalian cells comprising retroviral-mediated transduction of pathologic cells with a nucleic acid encoding a gene product having tumor suppressive  
35 function. The methodology provided involves the

introduction of a stably expressed tumor suppressor gene into a heterogeneous cell preparation (containing both normal and pathologic hyperproliferative cells) and, under suitable conditions, selectively transducing phenotypically pathologic hyperproliferative cells, suppressing the pathologic phenotype and reinfusing the treated cell preparation into the patient. Also provided by this invention is a method for treating a pathology in a subject caused by the absence of, or the presence of a pathologically mutated tumor suppressor gene.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the tumorigenicity of antibiotics-selected K562 cells in nude mice. K562 cells were infected with the p53-RV or NCV and selected in hygromycin as described in the legend to Table 3. (A)  $5 \times 10^6$  K562/p53 or K562/NCV (B)  $1 \times 10^7$  K562/p53 or K562/NCV were injected subcutaneously into opposite flanks of athymic Balb/c nu/nu mice. The mice were purchased from Simonsen Laboratories, Inc. (Gilroy, CA) and maintained in a pathogen-free environment. Once tumors were formed, they were measured weekly until the experiments were terminated.

Figure 2 shows delayed tumor formation in nude mice induced by K562 cells following a short-term infection with the p53-RV. K562 cells were infected with the p53-RV or NCV for 4 hours as described in Example II. The viral supernatant was removed and the cells were injected immediately into nude mice as described in Example III.

Figure 3 shows delayed tumor formation in nude mice induced by three human cell lines following short-term infections with the p53-RV. The three human cell lines, H69 (human small-cell lung carcinoma), H128 (human small-cell lung carcinoma) and HTB9 (human bladder carcinoma),

were obtained from the American Type Culture Collection (ATCC), (Rockville, MD). The short-term infections using either p53-RV or NCV were performed as described in Examples II and III.

5           Figure 4 shows lack of toxicity of the p53-RV viral supernatant on normal murine bone marrow cells at a high multiplicity. Normal mouse bone marrow cells were obtained from the femurs of Balb/c mice. The cells were isolated by ficoll-hypaque gradient, and infected with the  
10   p53-RV or NCV at MOI=1(A) or MOI=10(B) for 4 hours. At the end of infection, the viral supernatant was removed and the cells were seeded in 96-well plates at the density of  $5 \times 10^4$  cells per well containing murine GM-CSF ranging from 0 to 400 units/ml. The cells were incubated for 3 days and the  
15   number of viable cells were determined by the MTT assay as described by Mossman, T., J. Immunol. Methods, 65:55-63 (1983).

          Figure 5 graphically depicts the fraction of mice surviving with and without treatment with p53RV. Fifty  
20   SCID mice were each injected with  $25 \times 10^6$  K562 cells. Within 50 days post-injection, leukemia developed in the mice. After day 50, the mice were separated and treated with  $2.6 \times 10^5$  p53RV and  $2.6 \times 10^5$  heat-inactivated p53RV, by the i.p. method.

#### 25           DETAILED DESCRIPTION OF THE INVENTION

          There are approximately 5,000 bone marrow transplantations (BMT) each year (The BBI Newsletter, 156 (1991)). Most of these are performed on leukemia and lymphoma patients. A growing number of BMT are being done  
30   to support more intensive therapeutic approaches to breast and lung cancers, as well as for other indications (Droz, J.P. Eur. J. Can., 27:831-35 (1991); Menichella, G. Br.J. Haem., 79:444-50 (1991); Osbourne, C.K. Breast Can. Res.



Trtmt., 20:511-14 (1991)). Approximately 30% of these patients are candidates for tumor suppressive gene therapy. This number derives from the observation that about 30% of cancer patients either do not express the tumor suppressor gene or express an inactivated form of the tumor suppressor protein (Hollstein, M. et al., supra) . The preferred embodiments detailed below support the efficaciousness of a retrovirus encoding the human wild type tumor suppressor gene, p53-RV, in reversing the malignant phenotype of several leukemia and lymphoma cell lines as measured by abrogation or substantial inhibition of colony formation in soft agar assays, and as judged by reversing/inhibiting the ability of tumor cells to grow in nude mice following introduction of the wild type p53 gene.

For the K562 tumor cell line, which is derived from a chronic myelogenous leukemia patient in blast crisis (Andersson, L.C. et al., Int. J. Can., 23:143-7 (1979)) for two human small-cell lung carcinoma cell lines (H69 and H128) (Gazdar, A.F. et al., Can. Res., 40(10):3502-7 (1980)), and for one transitional cell (bladder) carcinoma cell line (HTB9) (Takahashi, R. et al., PNAS USA, 88:5257-61 (1991)) tumor suppression by p53 can be accomplished with a protocol involving short-term infections with the p53-RV. This protocol is completely consistent with current clinical methodology used in the preparation of bone marrow or peripheral blood hematopoietic cells for autologous bone marrow transplantation (ABMT) (Deisseroth, A.B. et al., Human Gene Therapy, 2:359-376 (1991)).

The present invention generally relates to an improved method of gene therapy for "negative purging" of pathologic hyperproliferative cells that contaminate preparations of autologous hematopoietic cells used for bone marrow reconstitution. As used herein, the term "hyperproliferative cells" includes but is not limited to cells having the capacity for autonomous growth, i.e.,

existing and reproducing independently of normal regulatory mechanisms. Hyperproliferative diseases may be categorized as pathologic, i.e., deviating from normal cells, characterizing or constituting disease, or may be categorized as non-pathologic, i.e., deviation from normal but not associated with a disease state. Pathologic hyperproliferative cells are characteristic of the following disease states, thyroid hyperplasia - Grave's Disease, psoriasis, benign prostatic hypertrophy, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, various leukemias and lymphomas. Examples of non-pathologic hyperproliferative cells are found, for instance, in mammary ductal epithelial cells during development of lactation and also in cells associated with wound repair. Pathologic hyperproliferative cells characteristically exhibit loss of contact inhibition and a decline in their ability to selectively adhere which implies a change in the surface properties of the cell and a further breakdown in intercellular communication. These changes include stimulation to divide and the ability to secrete proteolytic enzymes. The present invention will allow for high dose chemotherapy and/or radiation therapy, followed by autologous bone marrow reconstitution with hematopoietic cell preparations in which phenotypically pathologic cells have been reconstituted with a normal tumor suppressor gene. Application of the present invention will result in diminished patient relapses which occur as a result of reinfusion of pathologic hyperproliferative cells contaminating autologous hematopoietic cell preparations.

More specifically, the present invention relates to a method for depleting a suitable sample of pathologic mammalian hyperproliferative cells contaminating hematopoietic precursors during bone marrow reconstitution via the introduction of a stably-expressed wild type tumor

suppressor gene into the cell preparation (whether derived from autologous peripheral blood or bone marrow). As used herein, a "suitable sample" is defined as a heterogeneous cell preparation obtained from a patient, e.g., a mixed  
 5 population of cells containing both phenotypically normal and pathogenic cells. An example of a wild type tumor suppressor gene is the p53 gene, the coding sequence has been described by Chen et al. supra and is shown in Table 1.

10

TABLE 1

50

V\*SHR PGSR\* LLGSG DTLRS GWERA FHDGD TLPWI GSQTA FRVTA MEEPQ  
 100

SDPSV EPPLS QETFS DLWKL LPENN VLSPL PSQAM DDLML SPDDI EQWFT  
 15 150

EDPGP DEAPR MPEAA PPVAP APAAP TPAAP APAPS WPLSS SVPSQ KTYQG  
 200

SYGFR LGFLH SGTAK SVTCT YSPAL NKMFC QLAKT CPVQL WVDST PPPGT  
 250

20 RVRAM AIYKQ SQHMT EVVRR CPHHE RCSDS DGLAP PQHLI RVEGN LRVEY  
 300

LDDRN TFRHS VVVPY EPPEV GSDCT TIHYN YMCNS SCMGG MNRRP ILTII  
 350

TLEDS SGNLL GRNSF EVRVC ACPGR DRRTE EENLR KKGEP HHLEP PGSTK  
 25 400

RALPN NTSSS PQPKK KPLDG EYFTL QIRGR ERFEM FRELN EALEL KDAQA

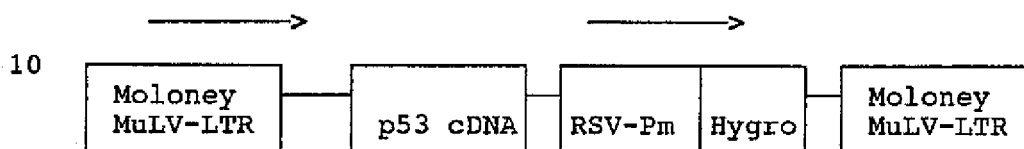
GKEPG GSRAH SSHLK SKKGQ STSRH KCLMF KTEGP DSD\*

\* = Stop codon

The preferred delivery system for the wild type  
 30 tumor suppressor gene is a replication-incompetent retroviral vector. As used herein, the term "retroviral" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the coding sequence into dividing cells. As used

herein, the terms "replication-incompetent" is defined as the inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of such vector is p53-RV, which has been described in detail by Chen et al. supra and is shown in Table 2.

TABLE 2  
General Schematic of p53-Retrovirus  
(p53-RV)



- Amphotropic Retrovirus
- 15 • Propagated in PA12 (3T3-derived) cells
- Titer ~ 1-3 X 10<sup>6</sup> Hygromycin Resistant CFU/ml

Another example of a replication-incompetent retroviral vector is LNL6 (Miller, A.D. et al., BioTechniques 7:980-990 (1989)). The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, P.H. et al., PNAS USA, 86:8912 (1989); Bordignon, C. et al., PNAS USA, 86:8912-52 (1989); Culver, K. et al., PNAS USA, 88:3155 (1991); Rill, D.R. et al., Blood, 79(10):2694-700 (1992)). Clinical investigations have shown that there are few or no adverse effects associated with the viral vectors (43: Anderson, Science, 256:808-13 (1992)). However, these methods have been limited to transfers of "gene markers" such as the neomycin gene that merely function as "tracking agents" for marking malignant cells before, and locating malignant cells after, reinfusion of bone marrow, however, the transduction of gene markers confers little clinical benefit to the affected patient who does not receive protection against subsequent relapse (Rill, D.R. et al. Blood, supra). The subject invention eliminates the necessity of the time

consuming procedure of transducing cell samples with a selectable marker gene, such as neomycin, to identify pathologic cells to facilitate subsequent attempts to remove those cells before reinfusion into the patient.

- 5           Other vectors are suitable for use in this invention and will be selected for efficient delivery of the nucleic acid encoding the tumor suppressor gene. The nucleic acid can be DNA, cDNA or RNA.

10           The subject invention provides a "shotgun" procedure whereby the cell sample is contacted with a retroviral vector in the absence of selective medium that does not necessarily contain a selectable marker gene, but notwithstanding, possesses the ability to simultaneously selectively target and transduce only the pathologic cell  
15           population in the heterogeneous cell preparation. Other methods of efficient delivery or insertion of a gene of interest into a cell are well known to those of skill in the art and comprise various molecular cloning techniques. As used herein, the terms "insertion or delivery" encompass  
20           methods of introducing an exogenous nucleic acid molecule into a cell.

          A variety of techniques have been employed in an attempt to deplete marrow of pathologic hyperproliferative cells before reinfusion, utilizing "purging" methods, e.g.,  
25           monoclonal antibodies or chemotoxins (Kaizer H. et al., Blood, 65:1504 (1985); Gorin, N.C. et al., Blood, 67:1367 (1986); De Fabritiis, P. et al., Bone Marrow Transplant, 4:669 (1989)). As used herein, the term "pathologic" includes abnormalities and malignancies induced by  
30           mutations and failures in the genetic regulatory mechanisms that govern normal differentiation that are not the result of gene loss or mutation. These techniques, however, have not resulted in reduced relapse rates, and have consistently resulted in damaging normal marrow progenitor

cells (Kaizer H. et al., supra; Gorin, N.C. et al., supra; De Fabritiis, P. et al., supra). The present invention addresses the aforementioned inadequacies and confers related advantages as well. These advantages include: (a) the use of a recombinant retroviral vector that does not require a selectable marker gene in combination with a short-term infection in the absence of selective medium eliminating the time consuming procedure traditionally employed to "selectively mark" the target cells before any "purging" of such cells is attempted, thereby dramatically reducing the time traditionally required for preparing hematopoietic cells for transplants; and (b) the retroviral mediated delivery methodology of the subject invention offers selective targeting of pathologic hyperproliferative cells in resting cultures of hematopoietic cells as a result of the higher infection frequency by the retroviral delivery system into actively dividing tumor cells (Miller et al., Mol. Cell. Biol., 10(8):4239-42 (1990)).

The ex-vivo introduction of a wild type tumor suppressor gene, via an efficient delivery system into pathologic hyperproliferative cells contaminating peripheral blood- or marrow-derived autologous hematopoietic cells will facilitate suppression of the hyperproliferative phenotype, by inducing transformation of the cell to a mature or benign phenotype or, alternatively, by inducing apoptosis or programmed cell death, thereby allowing patients receiving ABMT to have a longer, relapse-free survival. As used herein, the term "mature or benign cell" refers to the phenotypic characteristic of inability to invade locally or metastasize.

This invention further provides a method for transducing a pathologic hyperproliferative mammalian cell by contacting the cell with a suitable retroviral vector containing a nucleic acid encoding a gene product having a tumor suppressive function, under suitable conditions such

that the cell is transduced. In one embodiment, the gene product is expressed by a tumor suppressor gene and the tumor suppressor gene can be, but is not limited to wild type p53 gene, retinoblastoma gene RB, Wilm's tumor gene  
5 WT1 or colon carcinoma gene DCC. Additionally, the nucleic acid is DNA, cDNA or RNA.

The suitable conditions for contacting can be by infecting the sample cells in the absence of selective medium. "Suitable retroviral vector" has been defined  
10 above. This method is particularly useful when the pathological cells being contacted are prostate cells, psoriatic cells, thyroid cells, breast cells, colon cells, lung cells, sarcoma cells, leukemic cells or lymphoma cells.

15 The suitable time period for contacting can be less than about ten hours, or more specifically, about four hours. Transduction can be known to be complete, for example, when the hyperproliferative phenotype is characterized by the transduced cell expressing a mature or  
20 benign phenotype or by apoptosis or death of the transduced cell. This method has been shown to reduce tumor formation or tumorigenicity in a subject.

This method can be practiced ex vivo or in vivo. The practice of the ex vivo method is described above.  
25 When the method is practiced in vivo, the retroviral vector can be added to a pharmaceutically acceptable carrier and systemically administered to the subject. In one embodiment, the subject is a mammal, such as a human patient. Acceptable "pharmaceutical carriers" are well  
30 known to those of skill in the art and can include, but not be limited to any of the standard pharmaceutical carriers, such as phosphate buffered saline, water and emulsions, such as oil/water emulsions and various types of wetting agents.

As used herein, the term "administering" for in vivo purposes means providing the subject with an effective amount of the vector, effective to inhibit hyperproliferation of the target cell. Methods of administering pharmaceutical compositions are well known to those of skill in the art and include, but are not limited to, microinjection, intravenous or parenteral administration. Administration can be effected continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the vector used for therapy, the purpose of the therapy, the cell or tumor being treated, and the subject being treated.

The following examples are intended to illustrate, not limit this invention.

#### EXAMPLE I

Introduction of the p53-RV into leukemia or lymphoma-derived cell lines suppresses the malignant phenotype as measured by colony formation in soft agar

The retroviral vector carrying the human wild type p53-cDNA has been described (Chen et al. supra). p53-RV, an amphotropic retrovirus, is capable of infecting a wild range of human cell types (see below). This feature provides an advantage for ex vivo therapy of human leukemias, because the viral vector can deliver the wild type p53-cDNA into a number of different leukemic or other cell types, including tumor cells from solid tumors which may metastasize to marrow. The results of soft-agar assays using three leukemia or lymphoma cell lines following viral infection and antibiotic selection are shown in Table 3. In all three cases (HL-60, acute promyelocytic leukemia, p53-negative; Hut 78, acute T cell lymphoma, p53-negative; and Molt 3, acute lymphoblastic leukemia, mutant p53-positive) the introduction of wild type p53 by the p53-RV



resulted in either a reduction or elimination of colony formation in soft agar.

TABLE 3

	Cell Line	No. of Cells Seeded	Plating Efficiency
5	HL-60	$5 \times 10^5$	TMTc
	HL-60/T*	$5 \times 10^5$	4.7%
	HL-60	$10^5$	43%
	HL-60/T*	$10^5$	0%
10	HL-60	$5 \times 10^4$	55%
	HL-60/T*	$5 \times 10^4$	0%
	Hut 78	$10^5$	9.4%
	Hut 78/I#	$10^5$	0.39%
	Hut 78	$5 \times 10^4$	9.2%
	Hut 78/I#	$5 \times 10^4$	0%
15	Molt 3	$10^5$	11.7%
	Molt 3/I#	$10^5$	1.5%

\*Transfected

#Infected

The human leukemic cell lines, HL-60, Hut 78 and Molt 3, were obtained from American Type Culture Collection (ATCC). The cell lines Hut 78 and Molt 3 were infected with the p53-RV and the HL-60 cell line was transfected with p53-RV DNA. The p53-RV containing the wild type p53 cDNA isolated from human fetal brain and Moloney murine leukemia viral vector has been described by Chen et al., supra. This virus also carries the hygromycin resistant gene whose expression is driven by the Rous sarcoma virus (RSV) promoter sequence. The murine NIH3T3-derived packaging cell line, PA12 (Chen et al., supra), produces the p53-RV with titers ranging from  $1 \times 10^5$  to  $1 \times 10^6$  virus per ml.

Viral infections were carried out overnight in the presence of 4 $\mu$ g/ml polybrene. At the end of infections, 4 ml of fresh media were added to 2 ml of each infection mixture. The cells were selected in the presence  
5 of 400 $\mu$ g/ml hygromycin 2 days after infection.

Infected cells grew to confluency in 2-3 weeks following hygromycin selection. For the soft-agar assay, the cells were seeded in 6-well plates at the cell densities ranging from 10<sup>3</sup> to 10<sup>5</sup> in 0.33% agar as  
10 described (Chen et al., supra). Colonies consisting of more than 50 cells were scored 2 weeks later.

#### EXAMPLE II

Suppression of colony formation in soft  
agar by K562 (human chronic myelogenous leukemia) cells  
15 following a short-term infection with p53-RV

Mammalian cells infected with a retroviral vector carrying an antibiotic marker are usually pre-selected in vitro before testing for tumorigenicity in soft agar or in nude mice (Chen et al., supra, Cheng et al., supra).  
20 Because this process takes about three weeks, it would be cumbersome and expensive to pursue in the clinic. To mimic more closely the clinical situation in which tumor suppressor gene therapy may be applied during bone marrow purging, K562 cells were infected with p53-RV for four  
25 hours in vitro, then immediately tested their ability to form colonies in soft agar without any selection. A retroviral vector identical to p53-RV, but with the p53 coding sequence deleted, was used as a control (see Table 4). This vector is designated NCV (Negative Control  
30 Virus). As shown in Table 4, p53-RV decreased colony formation by infected K562 cells in a dose-dependent manner. At a multiplicity of infection, (MOI) of 1, the plating efficiencies of the p53 and NCV-infected cells were similar. However, at MOI of 3 and 10, there was a marked  
35 decrease in the plating efficiency of the p53-RV infected

cells. The plating efficiencies of the NCV-infected K562 cells were similar at all three multiplicities of infection. The latter result suggests that the dose-dependent reduction in tumor cell colony formation observed with increasing doses of p53-RV was due to introduction of the wild type p53. Furthermore, the result with NCV indicates that there is little non-specific toxicity associated with the retroviral infection up to MOI of 10, as measured by this assay.

10

TABLE 4

Virus Infection	No. of Cells Seeded	M.O.I.	Plating Efficiency (Colony No.)
p53-RV	10 <sup>4</sup>	1	3.10% (310)
		3	0.52% (52)
		10	0% (0)
Control RV	10 <sup>4</sup>	1	4.30% (430)
		3	5.30% (530)
		10	3.40% (340)
p53-RV	5X10 <sup>3</sup>	1	4.40% (220)
		3	1.80% (90)
		10	0.25% (13)
Control RV	5X10 <sup>3</sup>	1	4.70% (235)
		3	4.10% (205)
		10	6.50% (325)

15

Human chronic myelogenous leukemia (CML)-derived cell line, K562, was obtained from ATCC, Accession No. CCL243. To perform the short-term infections, K562 cells were infected with the p53-RV or NCV for 4 hours as described in Example I. Multiplicity of infection (MOI) was determined from the titer of the viral stocks and K562 cell number. At the end of infection, the viral supernatant was removed by pelleting the cells, and the concentrated cells were used immediately in the soft-agar assay as described in Example I.

25

To construct NCV, the plasmid containing the p53 viral genome was partially digested with BamHI, ligated, and used to transform *E. coli*. The plasmid with the deleted p53 gene was then selected by restriction enzyme analysis of mini-lysate DNA. This DNA was used to transfect/infect PsiCRIP packaging cell line as described (Danos, O. et al., PNAS USA, 85:6460-64 (1988)). The viral stock, termed negative control virus (NCV), was produced in PsiCRIP packaging cell line (56: Danos et al., supra) with a titer of about  $2 \times 10^5$  virus per ml.

#### EXAMPLE III

##### Tumorigenicity of K562 chronic myelogenous leukemia cells following infection by p53-RV and selection for hygromycin-resistant cells

To further broaden the efficacy experiments in relevant human tumor cell lines, K562 leukemic cells were infected with the p53-RV and hygromycin-resistant colonies (K562/p53) were tested for tumorigenicity in nude mice. When  $5 \times 10^6$  K562/p53 cells were injected subcutaneously into nude mice, no tumor formation was observed. In contrast, a comparable number of K562/NCV cells produced tumors in all five animals tested (Fig. 1A). When  $1 \times 10^7$  tumor cells were injected, the p53-RV infected cells produced visible tumors, although much smaller than those induced by the NCV-infected cells (Fig. 1B). It is likely that the lesions which formed on the flank of the animal injected with K562/p53 were induced by those cells which had lost expression of the wild type p53 gene (Johnson et al., supra). This conclusion is supported by the inability to detect p53 protein or transcripts in hygromycin selected clones after only a few passages in vitro (data not shown).

#### EXAMPLE IV

##### Tumorigenicity in nude mice of K562 cells following a short-term infection with p53-RV

To further assess the tumor suppressive activity

of the wild type p53 gene in K562 cells, and to determine whether a short-term infection protocol would be feasible for potential therapy of leukemias and lymphomas, K562 CML cells were co-incubated with p53-RV for four hours before testing for the malignant phenotype as determined by subcutaneous tumor formation in nude mice. Following a short-term infection by the p53-RV or the NCV, K562 cells were injected bilaterally into nude mice. In three separate experiments, substantial suppression of tumor formation on the flank injected with K562 exposed to the p53-RV was observed (Fig. 2).

#### EXAMPLE V

##### Growth suppressive activity of p53-RV on other human tumor cell types

While the major target for clinical trials consists of leukemia and lymphoma patients, other cancer patients are currently under consideration for clinical trials involving marrow reconstitution (Miller, C.W. et al., Can. Res., 52:1695-8 (1992); Takahashi, T. et al., Oncogene, 6:1775-8 (1991); Takahashi, T. et al., Science, 491-4 (1989)). Figure 3 demonstrates that short-term infections of two small-cell lung carcinoma cell lines (H69 in Fig. 3A; H128 in Fig. 3B) lead to substantial inhibition of tumor growth in nude mice. In addition, a similar experiment was performed with a human transitional cell (bladder) carcinoma cell line (HTB-9 in Fig. 3C). In contrast, tumor cells infected with NCV grow rapidly in this tumor model (Fig. 3A-C).

## EXAMPLE VI

Preliminary in vitro toxicity studies

A critical issue for clinical application of the p53-RV has to do with whether introduction of the p53 coding sequence under control of a murine retroviral LTR may inhibit proliferation of normal bone marrow cells. Preliminary studies suggest that such inhibition is not an issue in this system. To determine toxicity of the p53-RV, it was investigated whether exposure of normal bone marrow cells to p53-RV under conditions similar to those employed for a short-term infection of K562 leukemic cells would have an effect on the response of normal bone marrow cells to GM-CSF. A three-day proliferation assay and a methylcellulose colony forming assay using either murine or human normal bone marrow cells were employed to ascertain such response. Figure 5 shows that exposure of murine bone marrow cells at either a MOI of 1.0 or 10.0 has no effect on their proliferation in response to GM-CSF. In addition, when either human (Table 3) or murine (data not shown) bone marrow cells were tested in a GM-CSF dependent colony forming assay, no effect on normal marrow progenitor colony forming units following exposure to the p53-RV as compared to NCV or mock infected controls was observed.

Normal human bone marrow cells were isolated by ficoll-hypaque gradient. These cells were incubated with the p53-RV, NCV, or growth media in the presence of 4 $\mu$ g/ml polybrene for 4 hours. At the end of incubation, the cells were pelleted, and 1X10<sup>6</sup> cells per well were plated in 6-well plates containing 0.8% methylcellulose. Colonies larger than 50 cells per colony were scored 14 days later.

TABLE 5  
COLONY NUMBER

<-----rHuGMCSF----->			
Infection	None	0.02ng/ml	0.04ng/ml
Control	1	18	21
p53-RV(0.1)	0	16	28
NCV(0.1)	0	11	18
MOCK(0.1)	2	12	17
p53-RV(1.0)	2	15	23
NCV(1.0)	4	9	18
MOCK(1.00)	0	25	18

## EXAMPLE VII

15      Negative Purging of Small Cell Lung Cancer Cells (H69)  
          From a Preparation of Human Bone Marrow

Increasing quantities of small-cell lung cancer cell line H69 were added to human bone marrow cells. These cells were subjected to 3 two hour cycles of infection with p53-RV at a M.O.I. of 3. After infection the cells were pelleted and plated in methylcellulose. Colony formation is shown in Table 6. Suppression of tumor cell colony formation is evidenced in the p53-RV treated cultures, but is absent in the mock infected cultures. There is no evidence of suppression of bone marrow colony formation units in either case.

TABLE 6

Negative Purging of Small Cell Lung Cancer Cells (H69)  
From a Preparation of Human Bone Marrow

A. <u>Mock</u>					
5	<u>Colonies</u>	0	0.1	1.0	10.0 H69/HBMC%
	H69	0	53	100	475
	HBMC+Growth Factors	199	219	235	266
B. <u>P53-RV</u>					
10	<u>Colonies</u>	0	0.1	1.0	10.0 H69/HBMC%
	H69	0	1	11	181
	HBMC+Growth Factors	165	223	182	273
	HBMC+Growth	81	48	84	171
15	<ul style="list-style-type: none"> <li>• HBMC=normal human bone marrow cells</li> <li>• <math>5 \times 10^5</math> HBMC per well</li> <li>• HBMC + growth factors=total colony counts including H69, CFU-e, BFU-E, CFU-GEMM, and CFU-GM</li> <li>• Hyg=Hygromycin, 100 ug/ml</li> </ul>				

## EXAMPLE VIII

Mixing Experiment to Study "Bystander Effect"

Five (5)  $\times 10^7$  K562 cells (obtained from the American Type Culture Collection (ATCC)) were infected overnight with p53RV at a MOI equals 1 in RPMI medium containing 4 ug/ml polybrene (Sigma). Twenty-five nude mice were divided into 5 groups, with 5 animals per group. Every mouse in group 1 was infected subcutaneously with  $5 \times 10^6$  of p53RV infected cells. The ratio of infected to uninfected cells = 1:0. Every mouse in group 2 was injected subcutaneously with a mixture of  $2.5 \times 10^6$  infected cells and  $2.5 \times 10^6$  uninfected cells. The ratio of infected to infected cells = 1:1. Every mouse in group 3 was injected subcutaneously with a mixture of  $1.5 \times 10^6$  infected



cells and  $3 \times 10^6$  uninfected cells. The ratio of infected to uninfected cells = 1:2. Every mouse in group 4 was injected subcutaneously with a mixture of  $0.45 \times 10^6$  infected and  $4.5 \times 10^6$  uninfected cells (infected:uninfected = 1:10). All the mice in group 5 were injected subcutaneously with  $5 \times 10^6$  uninfected cells (infected:uninfected = 0:1). Nude mice were observed for tumor growth and survival time. Results of the study are summarized below.

10	Group	Ratio infected:uninfected	Tumor Formation	Survival Status <sup>b</sup>
	1	1:0	-	Alive and healthy at 200 days
	2	1:1	-	Alive and healthy at 200 days
	3	1:2	-	Alive and healthy at 200 days
	4	1:10	-	Alive and healthy at 200 days
15	5	0:1	+ <sup>a</sup>	All died within 90 days

a Measurable tumors developed by thirtieth day.

b Experiment was terminated on day 200, when all p53RV-selected animals were still alive and healthy.

This experiment shows that treatment with p53RV, even at a MOI of less than 1, inhibits tumor formation or "tumorigenicity" in nude mice.

#### EXAMPLE IX

5                    Effect of Intraperitoneal Injection  
                    of p53RV in K562 Bearing SCID Mice

Fifty (50) SCID mice were each injected i.p. with  $25 \times 10^6$  K562 cells (ATCC). Within 50 days post-injection, leukemia developed in the mice. The mice were then  
10 randomly separated into 3 groups and treated as outlined below:

Group 1: injected i.p. with RPMI media on day 50

Group 2: injected i.p. with 1 ml heat-inactivated p53RV  
15 (original titer =  $2.6 \times 10^5$  virus/ml, titer below detection limit after inactivation) on day 50

Group 3: injected i.p. with 1 ml p53RV ( $2.6 \times 10^5$  virus/ml) on day 50

Figure 5 shows that mice treated with p53RV survived over twice as long as mice treated with heat-  
20 inactivated virus or control mice. Thus, systemic treatment with the retroviral vector containing the tumor suppressor gene p53 reduced tumorigenicity in the mice and prolonged survival time.

Although the invention has been described with  
25 reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various  
modifications can be made without departing from the spirit  
30 of the invention. Accordingly, the invention is limited only by the following claims.

## WE CLAIM:

1. A method for transducing a pathologic hyperproliferative mammalian cell comprising contacting the cell with a suitable retroviral vector containing a nucleic acid encoding a gene product having a tumor suppressive function, under suitable conditions such that the cell is transduced.
2. The method of claim 1, wherein the gene product is expressed by a tumor suppressor gene.
3. The method of claim 2, wherein the tumor suppressor gene is wild type p53 gene, retinoblastoma gene RB, Wilm's tumor gene WT1 or colon carcinoma gene DCC.
4. The method of claim 1, wherein the suitable conditions are infecting the sample cells in the absence of selective medium.
5. The method of claim 1, wherein the suitable retroviral vector lacks a selectable marker gene.
6. The method of claim 1, wherein the suitable retroviral vector is replication-incompetent.
7. The method of claim 1, wherein the pathological cells are prostate cells, psoriatic cells, thyroid cells, breast cells, colon cells, lung cells, sarcoma cells, leukemic cells or lymphoma cells.
8. The method of claim 1, wherein the suitable time period is less than about ten hours.
9. The method of claim 8, wherein the time period is about four hours.

10. The method of claim 1, wherein suppressing the hyperproliferative phenotype is characterized by the transduced cell expressing a mature or benign phenotype.
11. The method of claim 1, wherein suppressing the  
5 hyperproliferative phenotype is characterized by apoptosis or death of the transduced cell.
12. The method of claim 1, wherein the contacting is effected ex vivo.
13. The method of claim 1, wherein the contacting is  
10 effected in vivo.
14. The method of claim 1, wherein the nucleic acid is RNA.
15. The method of claim 1, wherein the mammal is a human.
- 15 16. A method for treating a pathology in a subject caused by the absence of a tumor suppressor gene or the presence of a pathologically mutated tumor suppressor gene comprising administering to the subject an effective amount  
20 of a suitable retroviral vector containing a nucleic acid encoding a gene product having a tumor suppressive function, under suitable conditions.
17. The method of claim 16, wherein the gene product is expressed by a tumor suppressor gene.
18. The method of claim 17, wherein the tumor  
25 suppressor gene is wild type p53 gene, retinoblastoma gene RB, Wilm's tumor gene WT1 or colon carcinoma gene DCC.
19. The method of claim 16, wherein the suitable retroviral vector is replication-incompetent.

20. The method of claim 16, wherein the absence or presence of a pathologically mutated tumor suppressor gene causes a cell to hyperproliferate.
21. The method of claim 20, wherein the hyperproliferative cell is a prostate cell, a psoriatic cell, a thyroid cell, a breast cell, a colon cell, a lung cell, a sarcoma cell, a leukemic cell or a lymphoma cell.
22. The method of claim 21, wherein the treating of the hyperproliferative cell is characterized by apoptosis or death of the cell.
23. The method of claim 16, wherein the contacting is effected in vivo.
24. The method of claim 16, wherein the nucleic acid is RNA.

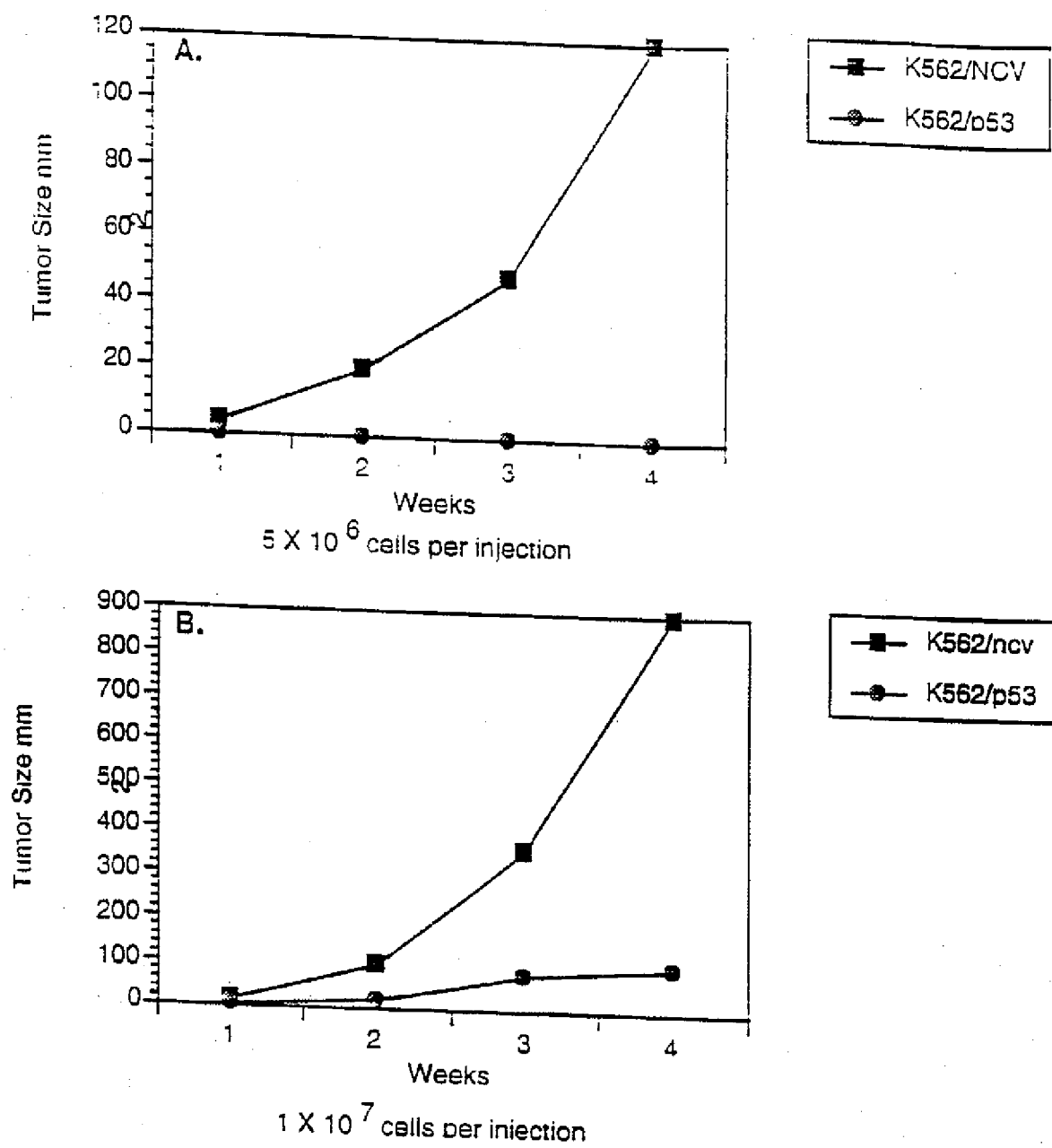


FIGURE 1

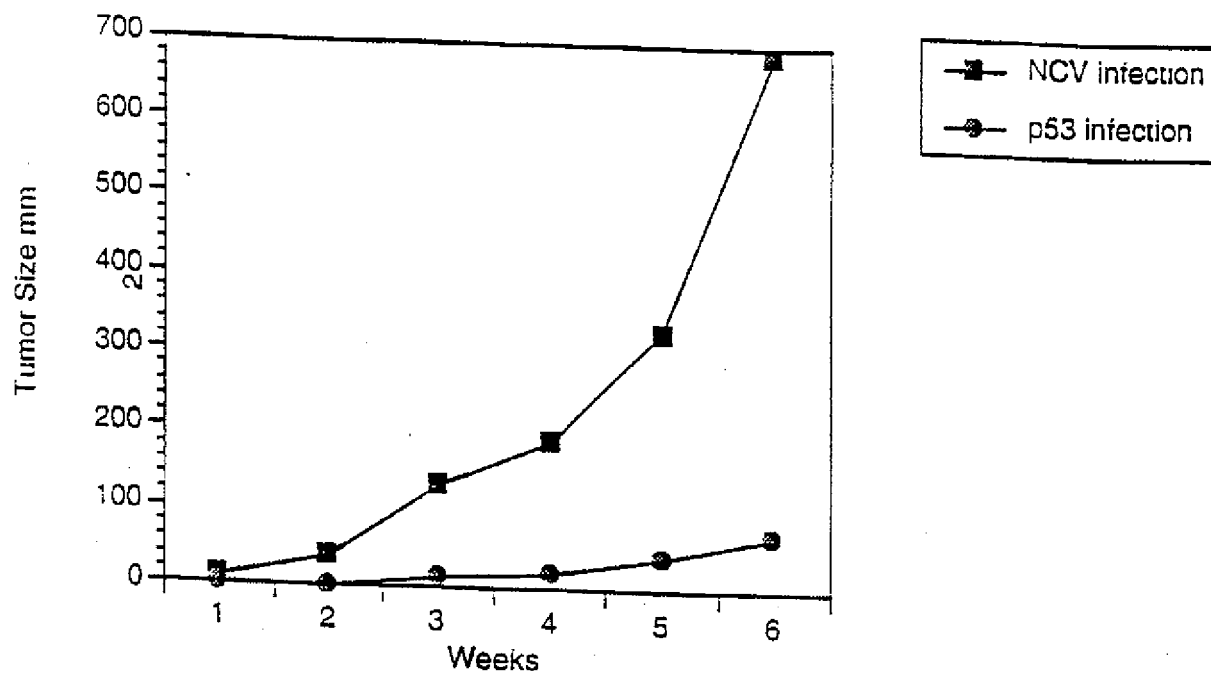


FIGURE 2

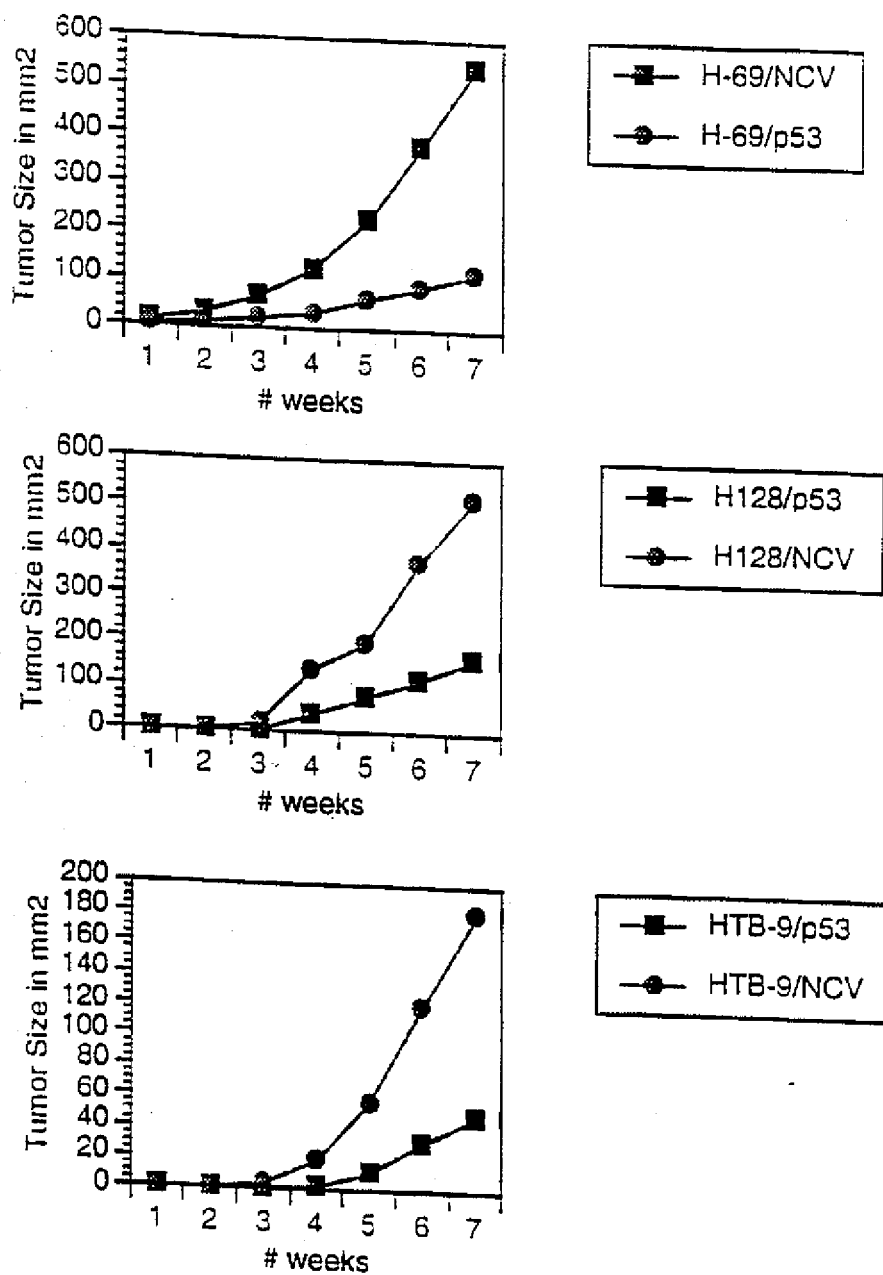
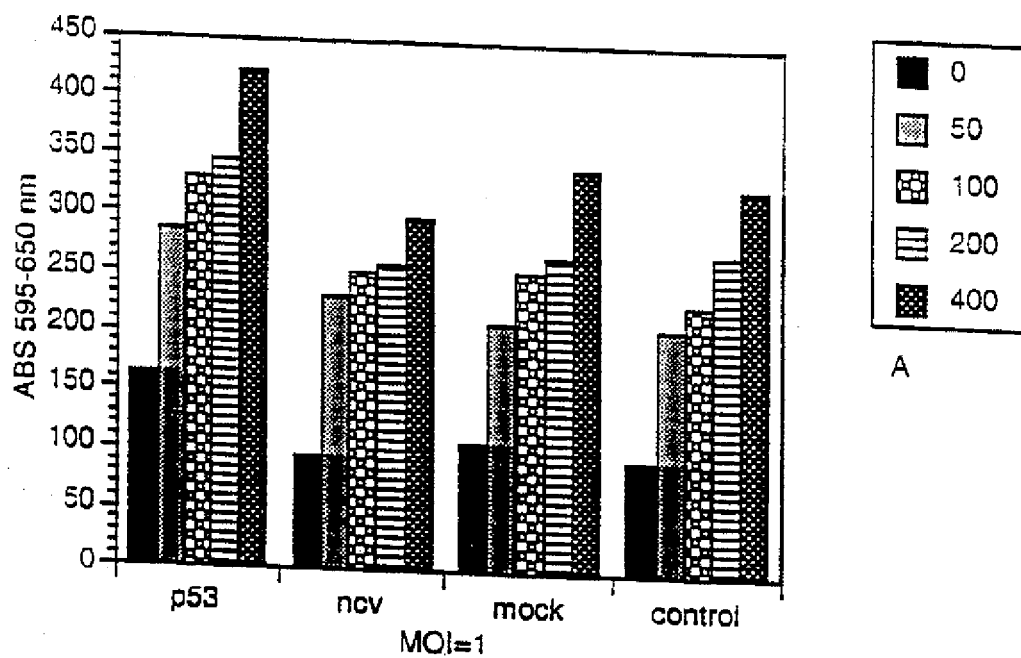
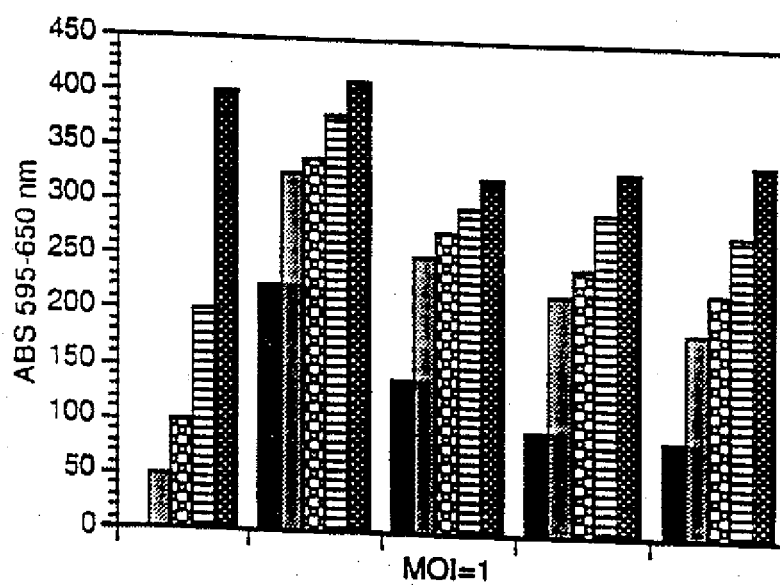


FIGURE 3





A



B.

FIGURE 4

## INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/US 93/08844

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/12 A61K48/00 C07K13/00 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 475 623 (UNIVERSITY OF CALIFORNIA) 18 March 1992 see the whole document ---	1-24
X	WO,A,91 15580 (RESEARCH DEVELOPMENT FOUNDATION) 17 October 1991 see the whole document ---	1-24
X	CANCER RESEARCH vol. 52, no. 1, 1 January 1992 pages 222 - 226 CHENG, J. ET AL. 'Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene' see the whole document ---	1-24
P,X	WO,A,93 10814 (VIAGENE INC.) 10 June 1993 see the whole document -----	1

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

17 December 1993

Date of mailing of the international search report

04-02-1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3916

Authorized officer

Chambonnet, F

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/08844

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 13, 16 - 24 and partially 1-12 and 14,15  
as far as they concern an in vivo method of treatment of the human/  
animal body the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such  
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 93/08844

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0475623	18-03-92	AU-A- 8262991	27-02-92
WO-A-9115580	17-10-91	AU-A- 7750191	30-10-91
		CA-A- 2079903	11-10-91
		CN-A- 1056427	27-11-91
		EP-A- 0527804	24-02-93
WO-A-9310814	10-06-93	AU-A- 3229793	28-06-93